Volume 290, number 1,2, 235–238 FEBS 10211 September 1991

ADONIS 001457939100922Z

Folding intermediates of hyperthermophilic D-glyceraldehyde-3-phosphate dehydrogenase from *Thermotoga maritima* are trapped at low temperature

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Received 8 July 1991

D-Glyceraldehyde-3-phosphate dehydrogenase from the hyperthermophilic eubacterium, *Thermotoga maritima*, is extremely thermostable showing a thermal transition beyong 105°C. At low temperature, 'cold denaturation' becomes detectable only in the presence of destabilizing agents. Reconstitution after preceding denaturation depends on temperature. At 0°C, no significant recovery of activity is detectable, whereas between 30 and 100°C reactivation reaches up to 85%. Shifting the temperature from low values to the range of optimum reconstitution releases the trapped intermediate in a fast reaction. Evidence from ultra-centrifugal analysis and far-UV circular dichroism proves the intermediate to be partially assembled to the tetramer, with most of its native secondary structure restored in a fast reaction. Fluorescence emission exhibits at least biphasic kinetics with the rate-limiting step(s) reflecting local adjustments of aromatic residues involved in tertiary contacts in the native state of the enzyme.

Cold denaturation; Folding intermediate; Glyceraldehyde-3-phosphate dehydrogenase; Thermophile; *Thermotoga maritima*

1. INTRODUCTION

D-Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12) from the hyperthermophilic eubacterium, *Thermotoga maritima*, exhibits intrinsic thermal stability with a denaturation temperature of the NAD^+ saturated enzyme beyond 105°C [1]. In the absence of denaturants, neither 'cold inactivation' nor subunit dissociation are detectable, even at low protein concentration. Correspondingly, the temperature dependence of the catalytic reaction yields only a slight curvature of the Arrhenius plot without indicating a significant break at low temperature. In denaturation/renaturation experiments, the enzyme (as its mesophilic counterparts) shows reversible denaturation/renaturation with reconstitution kinetics involving consecutive folding and association steps [2,3].

In the present study, the effect of temperature on the denaturation and reconstitution of GAPDH from *Thermotoga maritima* were investigated in order to determine the significance of the extreme physiological conditions on the acquisition of the native tertiary and quaternary structure of the enzyme. In contrast to previous findings which have shown that recombinant proteins from thermophilic microorganisms may be expressed in mesophilic hosts in active form [4–6], in vitro folding of the 'hyperthermophilic enzyme' turns out to be inhibited at low temperature. Under destabilizing conditions, the temperature profile of protein stability shows typical features of cold denaturation [7–9]. In reconstitution experiments, this observation allows kinetic intermediates to be detected at low temperature. Shifting the temperature from low to high values releases the trapped intermediate, thus allowing the kinetics of reactivation to be correlated with the regain of intrinsic spectral properties of the protein characteristic for its native three-dimensional structure.

Abbreviations: GAPDH, D-glyceraldehyde-3-phosphate dehydrogenase; GdmCl, guanidinium chloride; M_w, weight average molecular weight; $s_{20,w}$, sedimentation coefficient under standard conditions.

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2. MATERIALS AND METHODS

2.1. *Chemicals*

NAD^+ and glyceraldehyde-3-phosphate (barium salt), were purchased from Boehringer Mannheim; cysteamine was from Fluka (Buchs, Switzerland). Ultra-pure guanidinium chloride and urea were purchased from Schwarz-Mann (Orangeburg, NY), trypsin from Sigma (Deisenhofen). All other chemicals were A-grade substances from Merck (Darmstadt). Quartz-bidistilled water was used throughout. Buffer solutions were filtered and degassed 3 times.

Cultivation of Thermotoga maritima (MSB 8, DSM strain 3109), *enzyme purification* and *protein determination* were performed as reported previously [1].

Enzyme assays made use of the oxidation of glyceraldehyde-3-phosphate, monitoring the absorbance of NADH at 366 nm in an Eppendorf spectrophotometer at 40°C. The cuvettes were thermostatted and the actual temperature in the cuvettes monitored by a thermistor unit mounted in a reference cuvette. Standard assay mixtures contained 3

mM NAD^+, 10 mM di-sodium hydrogen arsenate and 5.2 mM glyceraldehyde-3-phosphate in 50 mM Tris-HCl buffer, pH 7.5, plus 5 mM EDTA and 5 mM cysteamine.

2.2. *Spectral analysis*

Ultraviolet absorption spectra were measured in Cary 1 and Perkin-Elmer Lambda 5 double-beam spectrophotometers using thermostatted quartz cuvettes equipped with a thermistor tele-thermometer. Fluorescence emission made use of a Perkin-Elmer luminescence spectrometer LS 5B. The effect of temperature on fluorescence emission was corrected making use of the tyrosine/tryptophan ratio known from the amino acid sequence [10]. Circular dichroism spectra were monitored in a Jasco J500A CD spectropolarimeter using thermostatted quartz cuvettes and a mean residue weight of 109.4 [10].

2.3. *Particle weights*

Sedimentation coefficients and molecular weights were determined in an analytical ultracentrifuge (Beckman, Palo Alto, CA; Spinco Model E) using double sector cells (30 mm) and scanning wavelengths at 280 and 230 nm.

2.4. *Stability*

Optimum long-term stability of holo-GAPDH was accomplished in 50 mM sodium phosphate buffer, pH 7.0, 5 mM EDTA, 5 mM cysteamine (standard phosphate buffer) or 50 mM Tris-HCl buffer, pH 7.0, 5 mM EDTA, 5 mM cysteamine (standard Tris buffer). The stability against denaturants was determined in aliquots incubated for at least 48 h given temperatures. Enzyme concentrations for activity and fluorescence measurements were 10–16 μg/ml, the one for CD measurement, 30–100 μg/ml.

2.5. *Denaturation/renaturation*

Denaturation was performed over a period of 120 min at 40°C in standard phosphate or Tris buffer in the presence of 6 mM GdmCl, renaturation at 0–100°C by dilution with GdmCl-free buffers. In order to exclude reactivation during the standard assay, trypsin was added in all stability and reconstitution experiments [11,12]. To reach the final state at low temperature, the reconstitution mixture was incubated at ≈3°C for 4–10 days. In order to accomplish reactivation, the temperature was shifted to 50°C. To normalize the reactivation kinetics, native controls (without denaturation) were kept under identical conditions with respect to temperature, incubation time and residual denaturant concentration. The kinetics of renaturation made use of fluorescence emission at 320 nm (λ_{exc} = 280 nm) and dichroic absorption at 222 nm. Temperature and solvent conditions were identical with those used in the reactivation experiments.

3. RESULTS AND DISCUSSION

Thermophilic adaptation of microorganisms is characterized by a shift of the growth curve to higher temperatures rather than its broadening [13]. Due to the flattened temperature profiles of the free energy of stabilization of 'thermophilic proteins' [8], the temperature shift does not imply that thermally hyperstable proteins necessarily exhibit cold denaturation in the accessible temperature range. In the case of GAPDH from *Thermotoga maritima*, the specific activity at low temperature vanishes without significantly deviating from linearity in the Arrhenius plot [1]. However, destabilizing the enzyme at low denaturant concentrations, reveals the typical parabolic temperature profiles indicating decreased stability at low *and* high temperatures (Fig. 1A). As in the case of the equilibrium transitions at high concentrations of chaotropic agents [3], deactivation is

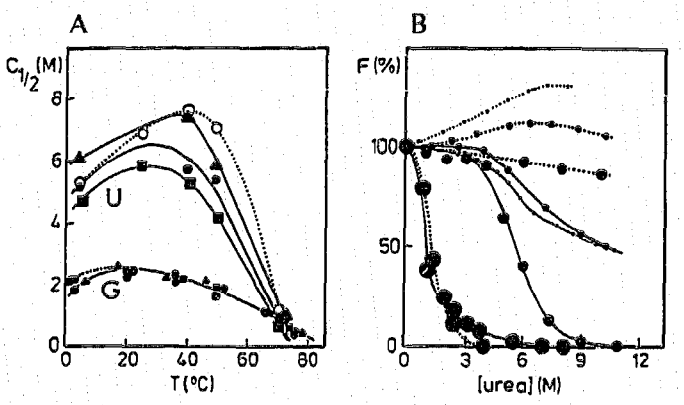


Fig. 1. Temperature- and urea-dependent unfolding transitions of GAPDH from *Thermotoga maritima* in standard phosphate (...) and Tris buffer, pH 7 (—), enzyme concentration 15–100 μg/ml. (A) Midpoints of guanidinium chloride (G) and urea (U) induced equilibrium transitions for the apo- (open symbols) and holoenzyme (closed symbols). Activity (□), fluorescence emission at 320 nm (○) and dichroic absorption at 222 nm (△). In the case of the urea-dependent denaturation of the holoenzyme in Tris buffer, the fluorescence and circular dichroism profiles refer to the first step of the bimodal equilibrium transition; due to the limited solubility of the denaturant, the complete N → D transition can only be detected at ≥50°C. (B) Effect of urea on the fluorescence emission at 5°C (●), 40°C (●), 50°C (●), 70°C (●), respectively.

found to precede denaturation (monitored by fluorescence emission and circular dichroism). The result may be taken to indicate that denaturation of the enzyme does not obey simple two-state behavior. However, the equilibrium transition may be affected by a variety of factors: (i) subunit dissociation and denaturation must not necessarily coincide; (ii) the NAD^+- and substrate binding domains may differ in their stability; (iii) specific ions and the coenzyme, NAD^+, may cause local stabilization, thus partially compensating chaotropic solvent effects. Fig. 1B clearly illustrates the complex superposition of stabilizing (NAD^+, phosphate) and destabilizing (Tris, high temperature) contributions. The inverse changes at 5–50°C in the presence of phosphate are compensated only at 70°C; in Tris-HCl at moderate temperature, the decrease in amplitude of the fluorescence change may be attributed to the uncoupling of the domains in the denaturation transition.

In 6 M GdmCl, denaturation of the enzyme is complete, independent of temperature and pH [3]. Under optimum conditions of long-term stability, high yields of reconstitution are observed at moderate temperature, neutral pH and low enzyme concentration (Fig. 2A). Hydrodynamic, spectral and catalytic properties prove the final product of renaturation to be enzyme in its native state. At low temperature (0°C), no significant regain of activity is detectable. At the upper limit of thermal stability (100°C), reactivation and heat denaturation run parallel; using the native protein as a reference, the yield of reconstitution still amounts to ≈30%.

In order to find out whether the enzyme in its inactive low temperature state is irreversibly inactivated or arre-

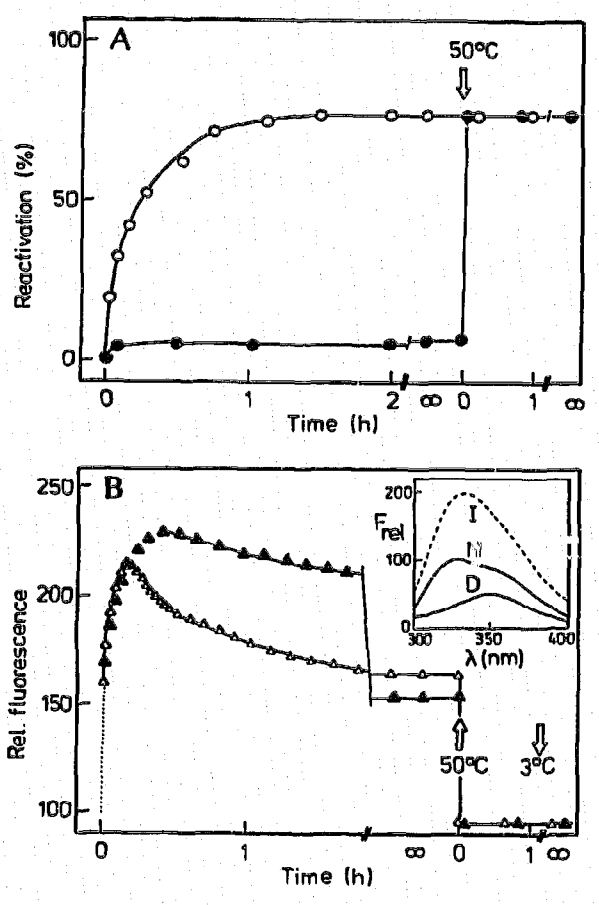
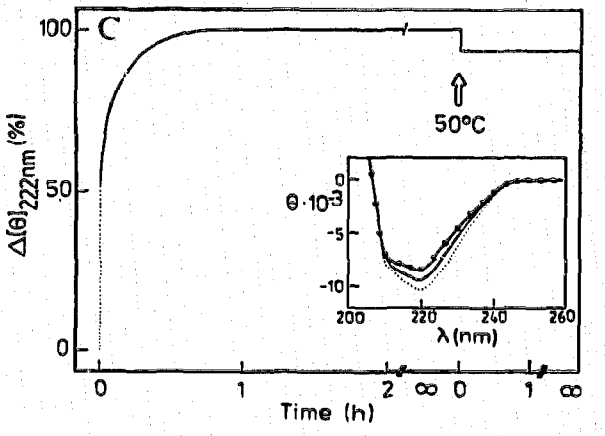


Fig. 2. Renaturation of holo-GAPDH from *Thermotoga maritima* after preceding denaturation in 6 M GdmCl, enzyme concentration μg/ml. After final yields were reached (arrow), the reactivation temperature in the 0°C sample were shifted to 50°C. (A) Reactivation in standard phosphate buffer pH 7 at 0°C (●) and 50°C (○). Activity measurements under standard assay conditions. (B) Recovery of native fluorescence emission at 320 nm (λ_{exc} = 280 nm) in standard phosphate (△) and Tris-HCl buffer, pH 7 (▲). Arrows mark temperature shifts to 50°C and 3°C, respectively. Relative fluorescence emission of the native control was set as 100%. (Insert) Fluorescence spectra of the native (N), denatured (D) and renatured enzyme in its intermediate state (I; obtained 12 min after starting renaturation). (C) Recovery of native dichroic absorption at 222 nm in standard Tris-HCl buffer, pH 7. Amplitude at 222 nm for the native enzyme at 40°C was set as 100%. (Insert) CD spectra of the native reference at 3°C (...) and the renatured enzyme at 3°C (—) and 50°C ((-●-). The decrease in amplitude reflects the incompleteness of renaturation; the spectra coincide after correcting for 82% yield.

sted as a stable intermediate, hydrodynamic and spectral analysis, as well as temperature shift experiments, were applied. As taken from ultracentrifugation, the trapped intermediate at 0°C and c = 30 μg/ml has regained the tetrameric quaternary structure ($s_{20,w}$ = 7.0 ± 0.5 S, M_w = 135 000); under meniscus depletion conditions (12 000 rpm), dissociation to the dimer and monomer is observed. The maximum of fluorescence emission of the intermediate is shifted from 350 to 327 nm. Including the fact that the far-UV circular dichroism spectrum of the refolded enzyme at 0°C shows exactly



the same characteristics (with only an insignificant decrease in amplitude compared to the native control), we may conclude that the reconstituted enzyme at 0°C is trapped in a native-like state.

Changing the temperature from 0°C to 50°C leads to a shift of the association equilibrium toward the tetramer (M_w = 140 500 ± 5000) accompanied by fast reactivation with a final yield identical to the one obtained in samples incubated at 50°C over the whole time period of reconstitution (Fig. 2A). Performing the same experiment using fluorescence emission at 320 nm, *renaturation* is found to show the same kinetic pattern. As shown in Fig. 2B, in a first phase the fluorescence intensity is strongly enhanced beyond the level observed for the native protein. Apparently, in this fast step the hydrophobic core is formed, with part of the aromatic residues still exposed to the solvent. Subsequently, a slow shuffling process leads back to the native state. The latter reaction is also detectable in the far-UV dichroic absorption (Fig. 2C), where the small increase in the amplitude at 222 nm can be fitted by first-order kinetics with a half-time of 7.2 min. There are no significant changes in helicity during the transition from the trapped intermediate to the native state.

4. CONCLUSIONS

Thermophilic adaptation of proteins may be brought about by slight modifications of a mesophilic amino acid sequence yielding a marginally increased free energy of stabilization (ΔG_{stab}), as well as broadening of the ΔG_{stab} vs temperature profile into the high-temperature range [8]. The expectation that hyperthermophilic GAPDH would exhibit cold denaturation is not confirmed. Obviously, the free energy parabola is flattened rather than shifted [15]. Low temperature destabilization becomes accessible only in the presence of chaotropic solvent components. Quantitative thermodynamic data cannot be obtained because of the wide range of extrapolation to zero denaturant concentration. Refolding of the enzyme after preceding denaturation in 6 M guanidinium chloride depends on temperature. As one

would expect from the fact that hyperthermophilic enzymes can be expressed in active form in mesophilic hosts [4–6], in vitro folding and association at elevated temperature (up to 100°C) may be easily accomplished. At 0°C, the enzyme is trapped as inactive, oligomeric folding intermediate with certain properties similar to those ascribed to the 'molten globule state' [14]. The corresponding characteristics are: (i) the native-like secondary structure; and (ii) the non-native environment of aromatic residues. As taken from the fact that correct quaternary contacts between the monomers are formed, the surface structure of the subunits must be well-defined and close to the native state. Sedimentation analysis proves that at low temperature the enzyme is present in a monomer–dimer–tetramer equilibrium. Upon shifting the temperature from 0°C to higher values, the trapped intermediate is rapidly transformed into the homogeneous native tetramer. In previous studies [2], it has been most difficult to obtain stable intermediates at the oligomer level. In the present case, the temperature dependence may open the way to look into the fast initial steps of folding and association using T-jump relaxation kinetics.

Acknowledgements: Thermotoga cells were a generous gift of Drs. K.O. Stetter and R. Huber. We thank P. Rehaber for fruitful discussions and G. Böhm and K. Lehle for kind assistance in programming and ultracentrifugation.

REFERENCES

[1] Wrba, A., Schweiger, A., Schultes, V., Jaenicke, R. and Závodsky, P. (1990) Biochemistry 29, 7584–7592.
[2] Jaenicke, R. (1987) Prog. Biophys. Mol. Biol. 49, 117–237.
[3] Schultes, V. (1991) Dissertation, University of Regensburg.
[4] Matsumura, M., Yasumura, S. and Aiba, S. (1986) Nature 323, 356–358.
[5] Imanaka, T., Shibazaki, M. and Takagi, M. (1986) Nature 324, 695–697.
[6] Fabry, S., Lehmacher, A., Bode, W. and Hensel, R. (1988) FEBS Lett. 237, 213–217.
[7] Jaenicke, R. (1990) Phil. Trans. Roy. Soc. 13, 326, 535–553.
[8] Jaenicke, R. (1991) Eur. J. Biochem. (in press).
[9] Privalov, P.L. (1990) Crit. Rev. Biochem. Mol. Biol. 25, 205–281.
[10] Schultes, V., Deutzmann, R. and Jaenicke, R. (1990) Eur. J. Biochem. 192, 25–31.
[11] Chan, W.W.-C., Mort, J.S., Chong, D.K.K. and MacDonald, P.D.M. (1973) J. Biol. Chem. 248, 2778–2784.
[12] Jaenicke, R. and Rudolph, R. (1986) Methods Enzymol. 131, 218–250.
[13] Jaenicke, R. (1981) Annu. Rev. Biophys. Bioeng. 10, 1–67.
[14] Jaenicke, R. (1991) Biochemistry 30, 3147–3161.
[15] Nojima, H., Hon-nami, K., Oshima, T. and Noda, H. (1978) J. Mol. Biol. 122, 33–42.